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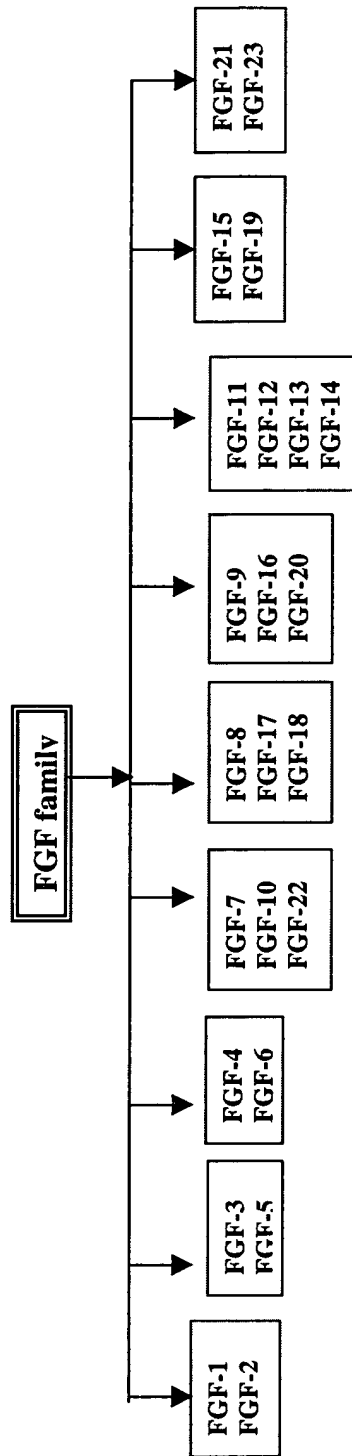
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Appendix I. Evolutionary Relationship of FGF superfamily





Appendix II. FGF receptor profile

| FGF family | FGFR1 IIIb | FGFR1 IIIc | FGFR2 IIIb | FGFR2 IIIc | FGFR3 IIIb | FGFR3 IIIc | FGFR4 |
|--------------|---------------|---------------|---------------|---------------|---------------|---------------|-------|
| FGF2 | + | ++ | - | + | - | ++ | ++ |
| FGF3 | + | - | + | - | - | - | - |
| FGF5 | - | + | - | +/- | - | - | - |
| FGF7 (KGF) | +/- | - | ++ | - | - | - | - |
| FGF10 (KGF2) | + | - | ++ | - | - | - | N/A |
| FGF22 | + | - | ++ | - | - | - | - |
| FGF9 | - | +/- | - | ++ | + | ++ | + |
| FGF20 | N/A | + | ++ | ++ | N/A | ++ | + |
| FGF4 | +/- | ++ | +/- | ++ | - | ++ | ++ |
| FGF8 | - | - | - | +/- | - | + | + |
| FGF23 | - | - | - | + | - | ++ | - |

Protein family review

Fibroblast growth factors

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Summary

Fibroblast growth factors (FGFs) make up a large family of polypeptide growth factors that are found in organisms ranging from nematodes to humans. In vertebrates, the 22 members of the FGF family range in molecular mass from 17 to 34 kDa and share 13-71% amino acid identity. Between vertebrate species, FGFs are highly conserved in both gene structure and amino-acid sequence. FGFs have a high affinity for heparan sulfate proteoglycans and require heparan sulfate to activate one of four cell-surface FGF receptors. During embryonic development, FGFs have diverse roles in regulating cell proliferation, migration and differentiation. In the adult organism, FGFs are homeostatic factors and function in tissue repair and response to injury. When inappropriately expressed, some FGFs can contribute to the pathogenesis of cancer. A subset of the FGF family, expressed in adult tissue, is important for neuronal signal transduction in the central and peripheral nervous systems.

Gene organization and evolutionary history

Gene organization

The prototypical *Fgf* genes contain three coding exons (Figure 1), with exon 1 containing the initiation methionine, but several *Fgf* genes (for example, *Fgf2* and *Fgf3*) have additional 5' transcribed sequence that initiates from upstream CUG codons [1,2]. The size of the coding portion of *Fgf* genes ranges from under 5 kb (in *Fgf3* and *Fgf4*) to over 100 kb (in *Fgf12*). In several *Fgf* subfamilies, exon 1 is subdivided into between two and four alternatively spliced sub-exons (denoted 1A-1D in the case of *Fgf8*). In these *Fgf* genes, a single initiation codon (ATG) in exon 1A is used. This gene organization is conserved in humans, mouse and zebrafish, but its functional consequences are poorly understood. Other subfamilies of *Fgfs* (such as *Fgf11-14*) have alternative amino termini, which result from the use of alternative 5' exons. It is not known whether a common 5'

untranslated exon splices to these exons or whether alternative promoter and regulatory sequences are used.

Most *Fgf* genes are found scattered throughout the genome. In human, 22 *FGF* genes have been identified and the chromosomal locations of all except *FGF16* are known (Table 1) [3-7]. Several human *FGF* genes are clustered within the genome. *FGF3*, *FGF4* and *FGF19* are located on chromosome 11q13 and are separated by only 40 and 10 kb, respectively; *FGF6* and *FGF23* are located within 55 kb on chromosome 12p13; and *FGF17* and *FGF20* map to chromosome 8p21-p22. These gene locations indicate that the *FGF* gene family was generated both by gene and chromosomal duplication and translocation during evolution. Interestingly, a transcriptionally active portion of human *FGF7*, located on chromosome 15q13-q22, has been amplified to about 16 copies, which are dispersed throughout the human genome [8].

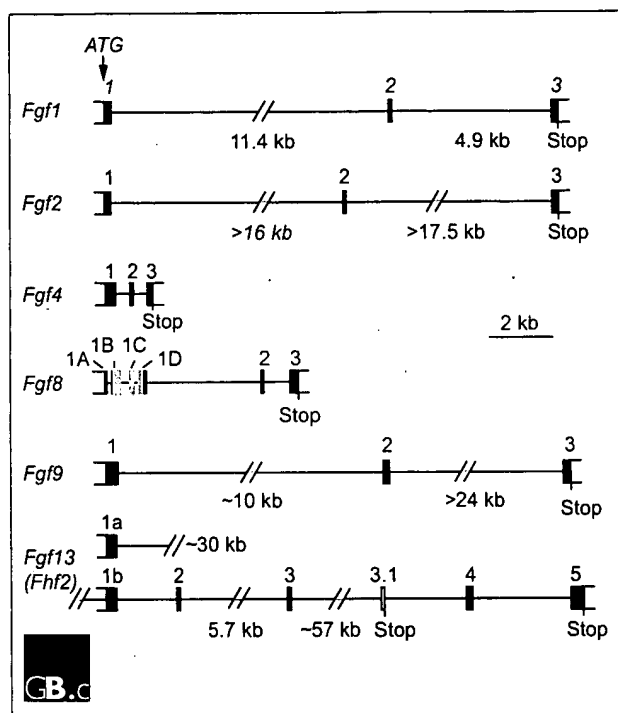


Figure 1

Gene structure of selected members of the *Fgf* family. Only the portion of each gene containing coding exons is shown. Constitutively expressed exons are in black; alternatively spliced exons are in gray. *Fgfs* 1, 2, 4 and 9 contain the prototypic three-exon organization. For *Fgf1*, 5' untranslated exons are not shown; inclusion of these exons extends the gene by approximately 69 kb [78]. *Fgf8* is an example of a gene with 5' alternative splicing, and *Fgf13* demonstrates alternatively used 5' exons separated by over 30 kb. References: *Fgf1* [78]; *Fgf2* [79]; *Fgf4* [80]; *Fgf8* [52]; *Fgf9* [81]; *Fgf13* [76].

In the mouse, there are at least 22 *Fgf* genes [3,9], and the locations of 16 have been identified (Table 1). Many of the mouse *Fgf* genes are scattered throughout the genome, but as in the human, *Fgf3*, *Fgf4* and *Fgf19* are closely linked (within 80 kb on chromosome 7F) and *Fgf6* and *Fgf23* are closely linked on chromosome 6F3-G1.

Evolutionary history

Fgfs have been identified in both invertebrates and vertebrates [3]. Interestingly, an *Fgf*-like gene is also encoded in the nuclear polyhedrosis virus genome [10]. *Fgf*-like sequences have not been found in unicellular organisms such as *Escherichia coli* and *Saccharomyces cerevisiae*. Although the *Drosophila* and *Caenorhabditis elegans* genomes have been sequenced, only one *Fgf* gene (*branchless*) has been identified in *Drosophila* [11] and two (*egl-17* and *let-756*) have been identified in *C. elegans* [12,13], in contrast to the large number of *Fgf* genes identified in vertebrates. The evolutionary relationship between invertebrate and vertebrate *Fgfs* is shown in Figure 2a.

The *Fgf* gene expansion has been hypothesized to be coincident with a phase of global gene duplications that took place during the period leading to the emergence of vertebrates [14]. Across species, most orthologous FGF proteins are highly conserved and share greater than 90% amino-acid sequence identity (except human FGF15 and mouse *Fgf19*; see below). To date, four *Fgfs* (*Fgf3*, 8, 17 and 18) have been identified in zebrafish, seven (*Fgf3*, *Fgf(i)*, *Fgf(ii)*, *Fgf8*, 9 and 20) in *Xenopus* (*Fgf(i)* and *Fgf(ii)* are most closely related to *Fgf4* and *Fgf6* [15]) and seven (*Fgf2*, 4, 8, 12, 14, 18 and 19) in chicken [3].

The apparent evolutionary relationships of the 22 known human FGFs are shown in Figure 2b. Vertebrate FGFs can be classified into several subgroups or subfamilies. Members of a subgroup of FGFs share increased sequence similarity and biochemical and developmental properties. For example, members of the FGF8 subfamily (FGF8, FGF17, and FGF18) have 70-80% amino acid sequence identity, similar receptor-binding properties and some overlapping sites of expression (for example, the midbrain-hindbrain junction) [16,17]. Members of FGF subgroups are not closely linked in the genome, however, indicating that the subfamilies were generated by gene-translocation or by genome-duplication events, not by local duplication events.

Human FGF15 and mouse *Fgf19* have not been identified. Human FGF19 is evolutionarily most closely related to mouse *Fgf15* (51% amino acid identity; Figure 2b) [18] and both the human FGF19 and mouse *Fgf15* genes are closely linked to the human and mouse *Fgf3* and *Fgf4* genes on orthologous regions of human chromosome 11q13 and mouse chromosome 7F (N.I., unpublished observations). These findings indicate that human FGF19 may be the human ortholog of mouse *Fgf15*. Because all other *Fgf* orthologs share greater than 90% amino acid identity, it remains possible that the true orthologs of these genes have not been identified, have been lost or have diverged during vertebrate evolution.

Characteristic structural features

FGFs range in molecular weight from 17 to 34 kDa in vertebrates, whereas the *Drosophila* FGF is 84 kDa. Most FGFs share an internal core region of similarity, with 28 highly conserved and six identical amino-acid residues [19]. Ten of these highly conserved residues interact with the FGF receptor (FGFR) [20]. Structural studies on FGF1 and FGF2 identify 12 antiparallel β strands in the conserved core region of the protein (Figure 3) [21,22]. FGF1 and FGF2 have a β trefoil structure that contains four-stranded β sheets arranged in a triangular array (Figure 3b; reviewed in [23]). Two β strands (strands β 10 and β 11) contain several basic amino-acid residues that form the primary heparin-binding site on FGF2. Regions thought to be involved in receptor binding are distinct from regions that bind heparin (Figure 3) [21-24].

Table 1

Chromosomal localizations of FGFs in human and mouse

| Human | | Mouse | | References | Accession numbers | |
|--------------|--------------|--------|----------|----------------------------------|------------------------|--------------------------------------|
| Gene | Location | Gene | Location | | Human | Mouse |
| FGF1 | 5q31 | Fgf1 | 18 | [82,83] | X65778, E03692, E04557 | U67610, M30641 |
| FGF2 | 4q26-27 | Fgf2 | 3A2-B | [84,85] | E05628, M27968 | M30644, AF065903, AF065904, AF065905 |
| FGF3 | 11q13 | Fgf3 | 7F | [86-88] | X14445 | Y00848 |
| FGF4 | 11q13.3 | Fgf4 | 7F | [87,89] | E03343 | M30642 |
| FGF5 | 4q21 | Fgf5 | 5E1-F | [85,90] | M37825 | M30643 |
| FGF6 | 12p13 | Fgf6 | 6F3-G1 | [91,92] | X63454 | M92416 |
| FGF7 | 15q15-21.1 | Fgf7 | 2F-G | [93,94] | M60828 | Z22703 |
| FGF8 | 10q24 | Fgf8 | 19C3-D | [54,95] | U36223, U56978 | Z48746 |
| FGF9 | 13q11-q12 | Fgf9 | 14D | [81,96,97] | D14838 | U33535, D38258 |
| FGF10 | 5p12-p13 | Fgf10 | 13A3-A4 | [98,99] | AB002097 | D89080 |
| FGF11 (FHF3) | 17p13.1 | Fgf11 | - | [100] | U66199 | U66203 |
| FGF12 (FHF1) | 3q28 | Fgf12 | 16B1-B3 | [31,100-102] | U66197 | U66201 |
| FGF13 (FHF2) | Xq26 | Fgf13 | X | [31,76,103] | U66198 | U66202, AF020737 |
| FGF14 (FHF4) | 13q34 | Fgf14 | 14 | [31] | U66200 | U66204 |
| - | - | Fgf15* | 7F | (N.I., unpublished observations) | - | AF007268 |
| FGF16 | - | Fgf16 | - | - | AB009391 | AB049219 |
| FGF17 | 8p21 | Fgf17 | 14 | [104] | AB009249 | AB009250 |
| FGF18 | 5q34 | Fgf18 | - | [105] | AB007422, AF075292 | AB004639, AF075291 |
| FGF19* | 11q13.1 | - | - | [106] | AB018122, AF110400 | - |
| FGF20 | 8p21.3-p22 | Fgf20 | - | [27,107] | AB030648, AB044277 | AB049218 |
| FGF21 | 19q13.1-qter | Fgf21 | - | [108] | AB021975 | AB025718 |
| FGF22 | 19p13.3 | Fgf22 | - | [109] | AB021925 | AB036765 |
| FGF23 | 12p13.3 | Fgf23 | 6F3-G1 | [7,75] (N.I., unpublished) | AB037973, AF263537 | AB037889, AF263536 |

*Human FGF19 and mouse Fgf15 may be orthologous genes.

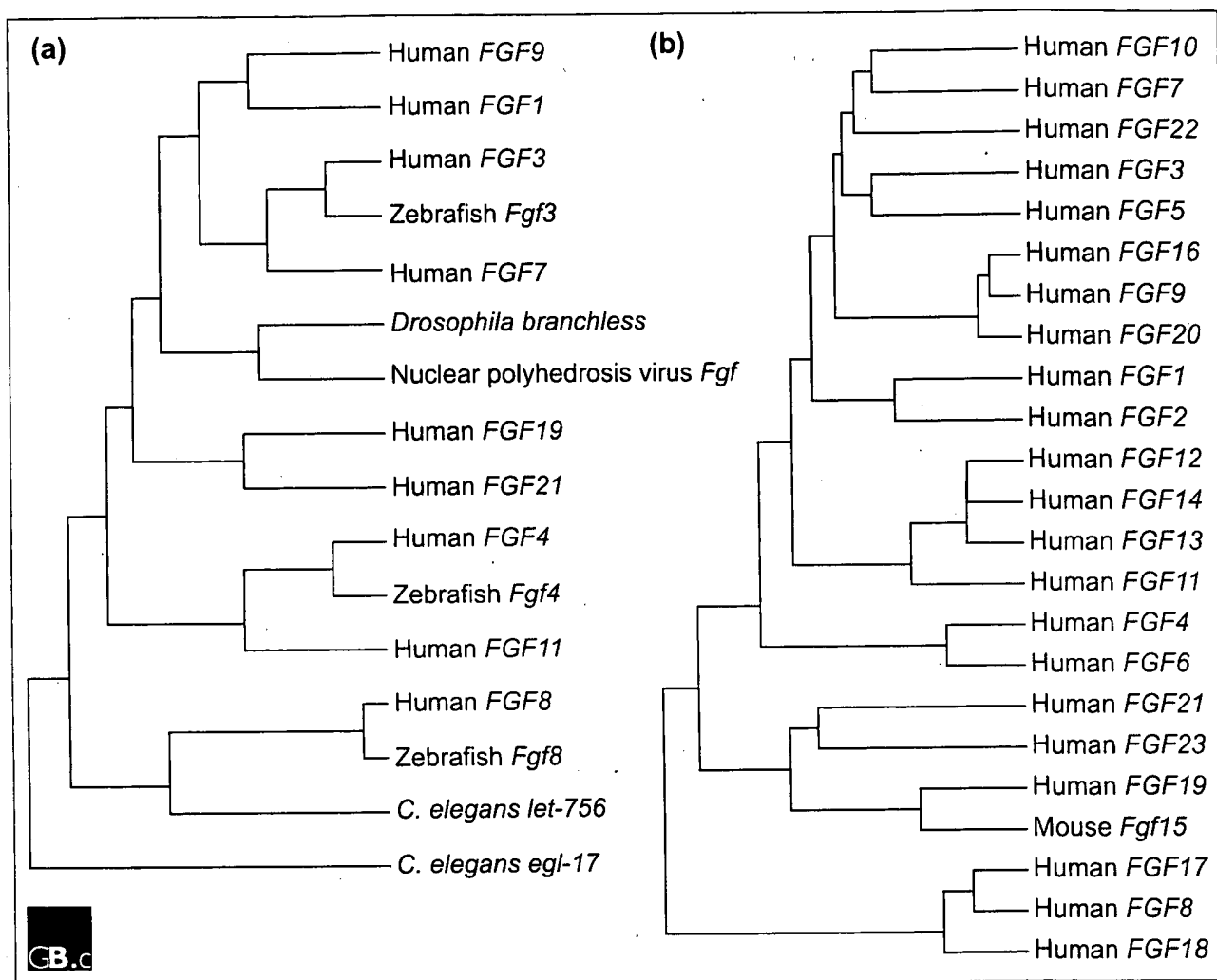
Localization and function

Localization

Subcellular localization and secretion

Most FGFs (FGFs 3-8, 10, 15, 17-19, and 21-23) have amino-terminal signal peptides and are readily secreted from cells. FGFs 9, 16 and 20 lack an obvious amino-terminal signal peptide but are nevertheless secreted [25-27]. FGF1 and FGF2 also lack signal sequences, but, unlike FGF9, are not secreted; they can, however, be found on the cell surface and within the extracellular matrix. FGF1 and FGF2 may be released from damaged cells or could be released by an exocytotic mechanism that is independent of the endoplasmic-reticulum-Golgi

pathway [28]. FGF9 has been shown to contain a non-cleaved amino-terminal hydrophobic sequence that is required for secretion [29,30]. A third subset of FGFs (FGF11-14) lack signal sequences and are thought to remain intracellular [31-34]. It is not known whether these FGFs interact with known FGFRs or function in a receptor-independent manner within the cell. FGF2 and FGF3 have high-molecular-weight forms that arise from initiation from upstream CUG codons [2,14,35]. The additional amino-terminal sequence in these proteins contains nuclear-localization signals, and the proteins can be found in the nucleus; the biological function of nuclear-localized FGF is unclear.

**Figure 2**

Evolutionary relationships within the FGF family. **(a)** Apparent evolutionary relationships between FGFs from vertebrates, invertebrates and a virus. Amino-acid sequences of nine representative FGFs were chosen from human and compared with FGFs from *Drosophila*, *C. elegans*, zebrafish and *Autographa californica* nuclear polyhedrosis virus. **(b)** Apparent evolutionary relationships of the 22 known human and murine FGFs. Sequences were aligned using Genetyx sequence analysis software and trees were constructed from the alignments using the neighbor-joining method.

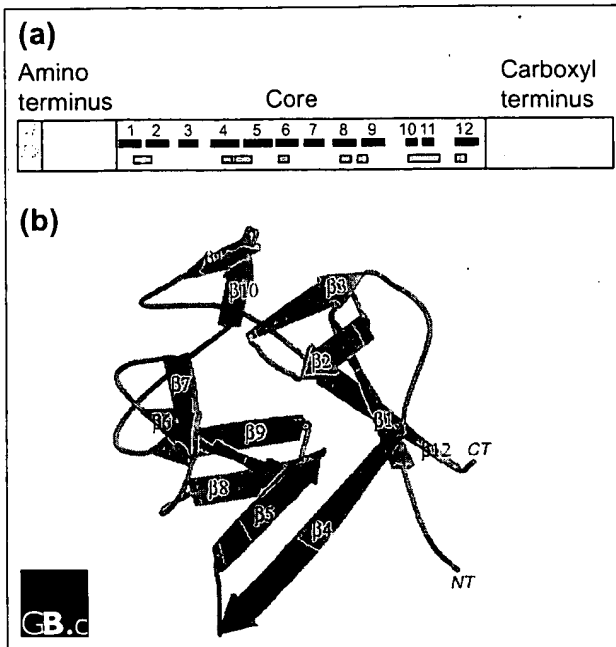
Developmental expression patterns and function

The 22 members of the mammalian FGF family are differentially expressed in many, if not all, tissues, but the patterns and timing of expression vary. Subfamilies of FGFs tend to have similar patterns of expression, although each FGF also appears to have unique sites of expression. Some FGFs are expressed exclusively during embryonic development (for example, *Fgf3*, 4, 8, 15, 17 and 19), whereas others are expressed in embryonic and adult tissues (for example, *Fgf1*, 2, 5-7, 9-14, 16, 18, and 20-23).

Function

The expression patterns of FGFs (see above) suggest that they have important roles in development. FGFs often signal

directionally and reciprocally across epithelial-mesenchymal boundaries [36]. The integrity of these signaling pathways requires extremely tight regulation of FGF activity and receptor specificity. For example, in vertebrate limb development, mesenchymally expressed *Fgf10* in the lateral-plate mesoderm induces the formation of the overlying apical ectodermal ridge; the ridge subsequently expresses *Fgf8*, which signals back to the underlying mesoderm [37]. This directional signaling initiates feedback loops and, along with other signaling molecules, regulates the outgrowth and patterning of the limb. Importantly, the differential expression of the alternative splice forms of the receptors in the apical ectodermal ridge and underlying-mesoderm is such as to limit or prevent autocrine signaling within a given compartment.

**Figure 3**

(a) Structural features of the FGF polypeptide. The amino terminus of some FGFs contains a signal sequence (shaded). All FGFs contain a core region that contains conserved amino-acid residues and conserved structural motifs. The locations of β strands within the core region are numbered and shown as black boxes. The heparin-binding region (pink) includes residues in the loop between β strands 1 and 2 and in β strands 10 and 11. Residues that contact the FGFR are shown in green (the region contacting Ig-domain 2 of the receptor), blue (contacting Ig-domain 3) and red (contacting the alternatively spliced region of Ig-domain 3). Amino-acid residues that contact the linker region are shown in gray [20]. **(b)** Three-dimensional structure of FGF2, a prototypical member of the FGF family. A ribbon diagram of FGF2 is shown; β strands are labeled 1-12 and regions of contact with the FGFR and heparin are color-coded as in (a) [22,24]. Image provided by M. Mohammadi.

Studies of the biochemical activities of FGFs have focused on the specificity of interactions between FGFs and FGFRs, on factors that affect the stability of FGFs and on the composition and mechanism of the active FGF-FGFR signaling complex.

Specificity of FGFs for FGF receptors

The FGFR tyrosine kinase receptors contain two or three immunoglobulin-like domains and a heparin-binding sequence [38-40]. Alternative mRNA splicing of the FGFR gene specifies the sequence of the carboxy-terminal half of immunoglobulin-domain III, resulting in either the IIIb or the IIIc isoform of the FGFR [41-43]. This alternative-splicing event is regulated in a tissue-specific manner and dramatically affects ligand-receptor binding specificity [44-48]. Exon IIIb is expressed in epithelial lineages and exon IIIc tends to be expressed in mesenchymal lineages [44,46-48]. *In vitro* patterns of binding specificity have been determined

for each splice form of FGFR1-3 and for FGFR4, which is not alternatively spliced [49-51]. Ligands specific for these receptor splice forms are expressed in adjacent tissues, resulting in directional epithelial-mesenchymal signaling. For example, epithelially expressed FGFR2b (that is, FGFR2 IIIb isoform) can be activated by FGF7 and FGF10, ligands produced in mesenchymal tissue [49-51]. These ligands show no activity towards mesenchymally expressed FGFR2c. Conversely, FGF8 is expressed in epithelial tissue and activates FGFR2c but shows no activity towards FGFR2b ([49,52] and our unpublished observations). Notably, FGF8 expression is often restricted to epithelial tissue such as the apical ectodermal ridge of the developing limb bud [53,54].

Interaction with heparin or heparan sulfate proteoglycans

An important feature of FGF biology involves the interaction between FGF and heparin or heparan sulfate (HS) proteoglycan (HSPG) [19]. These interactions stabilize FGFs to thermal denaturation and proteolysis and may severely limit their diffusion and release into interstitial spaces [55,56]. FGFs must saturate nearby HS-binding sites before exerting an effect on tissue further away, or else must be mobilized by heparin/HS-degrading enzymes. The interaction between FGFs and HS results in the formation of dimers and higher-order oligomers [57-59]. Although the biologically active form of FGF is poorly defined, it has been established that heparin is required for FGF to effectively activate the FGFR in cells that are deficient in or unable to synthesize HSPG or in cells pretreated with heparin/HS-degrading enzymes or inhibitors of sulfation [60-62]. Genetic studies have also shown that mutations in enzymes involved in HS biosynthesis affect FGF signaling pathways during development [19,63]. Additional studies have shown that heparin and/or HS act to increase the affinity and half-life of the FGF-FGFR complex (reviewed in [40,64]).

A minimal complex containing one FGF molecule per FGFR can form in the absence of HS [24]. Structural studies suggest that HS may bridge FGF2 and the FGFR by binding to a groove formed by the heparan-binding sites of both the ligand and the receptor [24,65]. Binding studies with soluble chimeric FGFRs have identified a second potential FGF-binding site that, in some cases, can interact cooperatively with the primary FGF-binding site [66].

Important mutants

Many members of the *Fgf* family have been disrupted by homologous recombination in mice. The phenotypes range from very early embryonic lethality to subtle phenotypes in adult mice. The major phenotypes observed in *Fgf* knockout mice are shown in Table 2. Because FGFs within a subfamily have similar receptor-binding properties and overlapping patterns of expression, functional redundancy is likely to occur. This has been demonstrated for *Fgf17* and *Fgf8*, which cooperate to regulate neuroepithelial proliferation in the mid-brain-hindbrain junction [17]. In the case of *Fgf* knockouts

resulting in early lethality, other functions later in development will need to be addressed by constructing conditional alleles that can be targeted at specific times and places in development. For example, *Fgf8*^{-/-} mice die by embryonic day 9.5 [67]. A conditional allele for *Fgf8* targeted to the apical ectodermal ridge has been used to demonstrate an essential role for *Fgf8* in early limb development [68,69].

Several mutations in *Fgf* genes have been identified in *C. elegans*, *Drosophila*, zebrafish, mouse and human. The *C. elegans* gene *egl-17* is required for sex myoblast migration [12], and a null allele of *let-756* causes developmental arrest of the early larva [13]. The *Drosophila branchless* gene is required for tracheal branching and cell migration [11]. In zebrafish, *acerebellar (ace)* embryos lack the cerebellum and the midbrain-hindbrain boundary organizer. The *ace* gene encodes the zebrafish homolog of *Fgf8* [70]. Interestingly, zebrafish *aussicht* mutant embryos, which overexpress *Fgf8*, also have defects in development of the central nervous system [71].

In the mouse, the *angora* mutation, which affects hair growth, was found to be allelic with *Fgf5* [72]. A mouse mutant with a Crouzon-syndrome-like craniofacial dysmorphism phenotype was found to result from an insertional

mutation in the *Fgf3/Fgf4* locus [73]. Recently, positional cloning of the autosomal dominant hypophosphataemic rickets gene identified missense mutations in human FGF23 [74]. A recent paper demonstrates that this disease is caused by a gain-of-function mutation [75]. The chromosomal location (Xq26) and tissue-specific expression pattern of *Fgf13* (also called *Fhf2*) suggests that it may be a candidate gene for Borjeson-Forssman-Lehmann syndrome, an X-linked mental retardation syndrome [76].

Frontiers

Issues most studied

FGFs have been intensely studied for nearly 30 years. Most of the early work focused on the mechanisms that regulate stability, secretion, export and interactions with heparin and on the mechanisms and consequences of signal transduction in various types of cells. More recent work has focused on the mechanisms regulating receptor specificity and receptor activation, the structure of the FGF-FGFR-HS complex, and the identification of new members of the FGF family. Functional studies have begun to address the role of FGFs in cell biology, development and physiology. Initial studies focused on the regulation of cell proliferation, migration and differentiation; more recent work has addressed the negative

Table 2.

FGF knockout mice

| Gene | Survival of null mutant* | Phenotype | References |
|---------------------------------|--------------------------|---|---|
| <i>Fgf1</i> | Viable | None identified | [110] |
| <i>Fgf2</i> | Viable | Mild cardiovascular, skeletal, neuronal | [110-114] |
| <i>Fgf3</i> | Viable | Mild inner ear, skeletal (tail) | [115] |
| <i>Fgf4</i> | Lethal, E4-5 | Inner cell mass proliferation | [116] |
| <i>Fgf5</i> | Viable | Long hair, angora mutation | [72] |
| <i>Fgf6</i> | Viable | Subtle, muscle regeneration | [117-119] |
| <i>Fgf7</i> | Viable | Hair follicle growth, ureteric bud growth | [120,121] |
| <i>Fgf8</i> | Lethal, E7 | Gastrulation defect, CNS development, limb development | [67,70,122,123] |
| <i>Fgf9</i> | Lethal, P0 | Lung mesenchyme, XY sex reversal | [124]; (J.S. Colvin et al., personal communication) |
| <i>Fgf10</i> | Lethal, P0 | Development of multiple organs, including limb, lung, thymus, pituitary | [125-127] |
| <i>Fgf12</i> (<i>Fhf1</i>) | Viable | Neuromuscular phenotype | (J. Schoorlemmer and M. Goldfarb, personal communication) |
| <i>Fgf14</i> (<i>Fhf4</i>) | Viable | Neurological phenotypes | (Q. Wang, personal communication) |
| <i>Fgf15</i> | Lethal, E9.5 | Not clear | (J.R. McWhirter, personal communication) |
| <i>Fgf17</i> | Viable | Cerebellar development | [17] |
| <i>Fgf18</i> | Lethal, P0 | Skeletal development | (N. Ohbayashi, Z. Liu, personal communication) |

*E, embryonic day; P, postnatal day.

effect of FGFs and FGFRs on proliferation of some cell types, which was surprising as FGFs were thought to promote proliferation. *In vitro* studies have now been complemented by gene targeting in mice. The knockout approach has been fairly successful in identifying primary phenotypes but will be challenged by the need to address redundancy amongst the 22 FGFs and to study their developmental and physiological functions after the point of lethality of the null allele.

Unresolved questions

A major unresolved question concerns the mechanism(s) regulating FGF activity *in vivo* in the presence of cell-surface and extracellular-matrix HSPG. Current hypotheses predict that tissue-specific heparan fragments of defined sequence (and particularly of defined sulfation pattern) will differentially regulate FGFs by controlling their diffusion in the extracellular matrix and their ability to activate specific receptors [77]. These issues will be resolved by determining the sequence of tissue-specific HS and by demonstrating whether specific HS sequences can modulate the binding specificity of FGFs beyond that determined by the specific FGFR and its alternative splice form in the presence of heparin.

A second area of research will aim to elucidate the developmental roles of all the FGFs, first alone and then in various combinations. This will include determining whether a single FGF with a defined developmental function interacts with one or multiple FGFRs. A third major frontier will be to elucidate the physiological roles of FGFs that are expressed in adult tissues. This will again involve testing combinations of FGFs in cases in which knockouts are viable and designing conditional alleles in cases of embryonic lethality. Major areas being considered include neuronal and cardiovascular physiology, neuronal regeneration and homeostasis and tissue repair.

The last major frontier will be to elucidate the primary roles of FGFs in genetic diseases and cancer. Several FGFs were initially cloned from human and animal tumors. Future work will be required to determine whether FGF activation is itself an etiological agent in primary human tumors or whether it is a progression factor in the pathogenesis of cancer. As functional roles for FGFs are elucidated in embryonic development, it is expected that various human birth defects and genetic diseases will be attributed to mutations in *Fgf* genes. These studies will probably lead to the development of pharmacogenetic agents to treat these diseases. Because a large number of skeletal diseases are caused by mutations in *Fgfr* genes, it is anticipated that mutations in some *Fgf* genes will also be involved in skeletal pathology.

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DNA sequences of FGFs listed in Table 1 can be accessed through this website.
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This database contains nomenclature and mapping information for human genes.
- Cytokine Family cDNA Database [<http://cytokine.medic.kumamoto-u.ac.jp/CFC/FGF/FGF.html>]
This database contains nomenclature and mapping information for the FGF family.
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Large induction of keratinocyte growth factor expression in the dermis during wound healing

(fibroblast growth factors/fibroblast growth factor receptors/gene expression/epidermis)

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ABSTRACT Recent studies have shown that application of basic fibroblast growth factor (basic FGF) to a wound has a beneficial effect. However, it has not been assessed whether endogenous FGF also plays a role in tissue repair. In this study we found a 160-fold induction of mRNA encoding keratinocyte growth factor (KGF) 1 day after skin injury. This large induction was unique within the family of FGFs, since mRNA levels of acidic FGF, basic FGF, and FGF-5 were only slightly induced (2- to 10-fold) during wound healing, and there was no expression of FGF-3, FGF-4, and FGF-6 detected in normal and wounded skin. High levels of FGF receptor 1 and FGF receptor 2 mRNA and low levels of FGF receptor 3 mRNA were found in both normal and wounded skin. No change in the levels of these transcripts was detected during wound healing. *In situ* hybridization studies revealed highest levels of KGF mRNA expression in the dermis at the wound edge and in the hypodermis below the wound. In contrast, mRNA encoding the receptor of this growth factor (a splice variant of FGF receptor 2) was predominantly expressed in the epidermis. These results suggest that basal keratinocytes are stimulated by dermally derived KGF during wound healing and implicate a unique role of this member of the FGF family in wound repair.

Cutaneous injury initiates a complex series of biological processes that are involved in wound healing. These processes involve many different cell types in migration, proliferation and differentiation, removal of damaged tissue, and production of extracellular matrices (1). Extensive histological studies have provided descriptive information on the cellular events involved in inflammation and tissue repair; however, little is known about the mediators that initiate and sustain wound repair.

The local application of platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) to wounds has been shown to accelerate dermal as well as epidermal wound healing (2–8). Therefore, it seemed likely that endogenous PDGF and fibroblast growth factor (FGF) are also involved in tissue repair. Whereas the induction of PDGF and PDGF receptor expression in wounds has been demonstrated (9), nothing is known about the role of endogenous FGF in wound healing. To gain insight into the contribution of FGFs *in vivo* to the healing of full-thickness wounds, we have investigated the mRNA expression of the seven members of the FGF family as well as their receptors (FGFR1–FGFR3) in normal skin and during wound healing. Our data show that acidic FGF (aFGF), bFGF, FGF-5, and particularly keratinocyte growth factor (KGF) are expressed in normal skin and induced upon injury. Whereas KGF was predominantly expressed in stromal cells below the wound and at the wound

edge, receptors for KGF (KGFR) were detected in the epidermis. This suggests that a KGF-mediated paracrine interaction may be important for the migration and proliferation of epidermal keratinocytes seen during wound healing. These data provide a molecular basis for understanding the mechanisms that contribute to tissue repair and imply an important role of KGF in wound healing.

MATERIALS AND METHODS

Animal Care. Mice were housed and fed according to the guidelines set forth by the Committee on Animal Research of the University of California, San Francisco.

Wounding and Preparation of Wound Tissue. Full-thickness excisional wounds were created along the backs of 50 adult BALB/c F₁ mice. Six wounds were created on each animal, and skin biopsy specimens from 10 animals were obtained at each of the following times: 12 hr, 1 day, 5 days, and 7 days after wounding. Biopsy of the 60 wounds from the 10 animals resulted in ≈ 4 cm² of tissue. A similar amount of skin was removed from the back of unwounded control animals. Tissue taken for RNA isolation was immediately frozen in liquid nitrogen. Tissue for *in situ* hybridization was fixed overnight in 4% paraformaldehyde in phosphate-buffered saline and subsequently frozen in OCT compound (Miles).

RNA Isolation and RNase Protection Assay. For RNA isolation, fresh skin biopsy specimens were frozen in liquid nitrogen and used for RNA isolation as described (10). For RNase protection mapping of FGF and FGFR transcripts, DNA probes were cloned into the transcription vector pBlue-script II KS(+) (Stratagene) and linearized. An antisense transcript was synthesized *in vitro* by using T3 or T7 RNA polymerase and [³²P]rUTP (800 Ci/mmol, Amersham; 1 Ci = 37 GBq). Samples of 50 μ g of total cellular RNA were hybridized at 42°C overnight with 100,000 cpm of the labeled antisense transcript. Hybrids were digested for 40 min at 30°C with RNases A and T1 as described (11). Protected fragments were separated on 5% polyacrylamide/8 M urea gels and analyzed by autoradiography. The same RNA preparations were used for all protection assays. The increase in FGF mRNA levels was quantitated by laser scanning densitometry of the autoradiograms.

DNA Templates. FGFs were as follows: 227-base-pair (bp) fragment corresponding to nucleotides 242–469 of the murine aFGF cDNA (12); 239-bp fragment corresponding to nucleotides 175–414 of the murine bFGF cDNA (12); 276-bp fragment corresponding to nucleotides 1021–1297 of the murine Int-2 (mammary tumor integration site 2; FGF-3) cDNA (13); 343-bp fragment corresponding to nucleotides 267–610 of the

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Abbreviations: FGF, fibroblast growth factor; aFGF and bFGF, acidic and basic FGFs; KGF, keratinocyte growth factor; FGFR, FGF receptor; KGFR, KGF receptor.

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murine Hst (human stomach cancer; FGF-4) cDNA (12); 303-bp fragment corresponding to nucleotides 1–303 of the murine FGF-5 cDNA (12); 304-bp fragment corresponding to nucleotides 406–710 of the murine FGF-6 cDNA (14); 201-bp fragment corresponding to nucleotides 23–224 of the murine KGF cDNA (F.F.-P. and C. Dickson, unpublished data).

FGF receptors were as follows: 361-bp fragment corresponding to nucleotides 793–1154 of the murine FGFR1 cDNA (15). This fragment encodes the complete extracellular third immunoglobulin-like domain of the published murine FGFR1 sequence (15); 298-bp fragment corresponding to nucleotides 2140–2438 of murine FGFR2 cDNA (16). This fragment encodes the amino-terminal end of the first kinase domain and the kinase insert; 430-bp fragment corresponding to nucleotides 1233–1663 of the murine FGFR3 cDNA (D. Ornitz and P. Leder; personal communication). This fragment encodes sequences from the middle of the third immunoglobulin-like domain of murine FGFR3 to the beginning of the first kinase domain.

In Situ Hybridization. Antisense riboprobes were made by *in vitro* transcription with T3 and T7 RNA polymerases and ³⁵S-labeled UTP as described (11). Plasmids encoding the transmembrane region of FGFR1, the kinase I/kinase insert region of FGFR2, and the coding sequences of KGF (see above) were used as templates. For *in situ* hybridization, skin and wound tissues were prepared as described (17). *In situ* hybridization was performed on frozen sections as described (18). After hybridization, sections were coated with NTB2 nuclear emulsion (Kodak) and exposed in the dark at 4°C for 18 days. After development, the sections were counterstained with hematoxylin/eosin.

RESULTS

mRNA Expression of FGFRs in Normal and Wounded Skin.

To investigate the mRNA expression of FGFRs in normal and wounded skin, we isolated RNA from excisional wounds at different intervals after wounding and performed RNase protection assays. For each time point, 60 wounds from 10 mice were excised and used for RNA isolation. Normal skin from nonwounded mice was used as a control. mRNA encoding three different FGF receptors (FGFR1, FGFR2, and FGFR3) was detected in normal and wounded skin (Fig. 1). Whereas FGFR1 and FGFR2 were expressed at high levels, only low levels of FGFR3 mRNA were found. This is based on the finding that the protection assays with the FGFR1 and FGFR2 probes were exposed for 16 hr, whereas the protection assay with the FGFR3 probe was exposed for 4 days. No change in the mRNA levels of FGFR1 and FGFR2 was detected during the process of wound healing. FGFR3 mRNA was slightly induced within 5 days after injury.

mRNA Expression of FGFs in Normal and Wounded Skin.

To investigate the mRNA expression of different FGFs in normal and wounded skin, we performed RNase protection assays with the same RNA preparations that had been used for the expression analysis of FGFR transcripts (see above). Consistent with recent findings (19), mRNA encoding KGF was detected in normal skin (Fig. 2). During wound healing the expression of this mRNA was considerably increased (Fig. 2); the expression level of KGF transcripts was induced 9-fold within 12 hr after wounding and 160-fold within 24 hr (Fig. 3). In 7-day wounds, KGF mRNA levels were still 100-fold higher compared with the unwounded basal level.

Relative to KGF expression, much lower levels of aFGF, bFGF, and FGF-5 were found in normal and wounded skin (see Fig. 3 and autoradiogram exposure times in the legend of Fig. 2). Expression of aFGF and FGF-5 was induced 10-fold and 2-fold, respectively, within 24 hr after wounding (Fig. 3). Expression of bFGF peaked at day 5 after wounding, and expression levels were 4-fold higher after this period compared with the basal level. In contrast to the prolonged

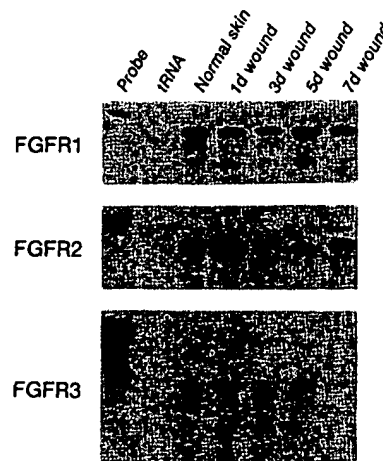


FIG. 1. Expression of FGFR mRNAs in normal and wounded skin. Total cellular RNA (50 μ g) from normal and wounded mouse back skin was analyzed by RNase protection assay by using probes that hybridize to mRNA encoding FGFR1 (Top), FGFR2 (Middle), and FGFR3 (Bottom). Hybridization was performed under high-stringency conditions to avoid cross-hybridization with other FGFR mRNAs. The 361-nucleotide FGFR1 probe was complementary to the coding sequences of the complete third immunoglobulin-like domain of the published murine FGFR1 (15). The 298-nucleotide FGFR2 probe was complementary to sequences encoding the first kinase domain and the kinase insert of murine FGFR2 (16). The 430-nucleotide FGFR3 probe was complementary to sequences encoding the amino-terminal end of the third immunoglobulin-like domain, the transmembrane region, and the extracellular and intracellular juxtamembrane regions. The FGFR1 and FGFR2 gels were exposed for 16 hr, and the FGFR3 gel was exposed for 4 days. The time in days (d; 1, 3, 5, and 7) after injury is indicated at the top of each lane. Each hybridization probe (200–2000 cpm) was added to the lanes labeled "probe." They were used as a size reference and do not reflect the amounts of probe (100,000 cpm) that were added to all hybridization mixtures.

induction of KGF expression, aFGF, bFGF, and FGF-5 transcripts returned to the basal value within 7 days after wounding (Fig. 3). No expression of FGF-3 (Int-2), FGF-4 (Hst), and FGF-6 mRNA was detected in normal or wounded skin (data not shown). These results show that several members of the FGF family are expressed during wound healing and that KGF is the predominant FGF induced during this process.

In Situ Hybridization of KGF and FGFR2 mRNA. To determine the site of expression of KGF and FGFR2 during wound healing, we performed *in situ* hybridization on normal skin and on 1-day-old wounds. Consistent with the protection assay data, a significant induction of KGF mRNA expression was observed within 1 day after wounding, and transcripts encoding this growth factor were expressed at high levels in some cells below the wound (Fig. 4D) and at the wound edge (not shown). The observation that only certain cells in the dermis and hypodermis express high levels of KGF suggests that these cells represent a distinct cell population that might have an important function in wound healing.

The receptor for KGF was recently shown to be a splice variant of FGFR2 (20). Using a probe that detects all membrane-spanning variants of FGFR2, including the KGFR, we found mRNA encoding FGFR2 predominantly in the epidermis (Fig. 4C). By RNase protection assay we found that the specific splice variant of FGFR2 that is known to bind KGF is the predominant form of FGFR2 in the skin (data not shown). Therefore, the FGFR2 transcripts that we detected in the epidermis predominantly encode KGFRs. The same expression pattern of KGF and KGFR was observed at later

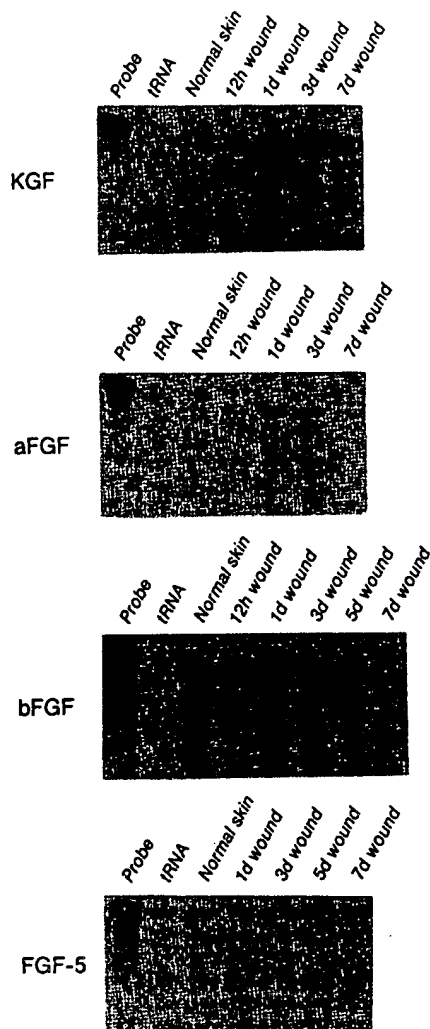


FIG. 2. mRNA expression of FGFs in normal and wounded skin. Total cellular RNA (50 μ g) from normal and wounded back skin was analyzed by RNase protection assay with RNA hybridization probes complementary to mRNA encoding (from top to bottom) KGF, aFGF, bFGF, and FGF-5. Hybridizations were performed under high-stringency conditions to avoid cross-hybridizations with other FGF mRNAs. The same RNA preparations were used for all hybridizations of Fig. 1 and Fig. 2. The gels were exposed for 12 hr (KGF), 3 days (FGF-5), or 5 days (aFGF and bFGF). The time after injury is indicated on top of each lane: 12h, 12 hours; 1d, 3d, and 7d, 1, 3, and 7 days.

stages of wound healing (days 3–7 after injury), with KGF being expressed in the dermis and KGFR in proliferating keratinocytes which are in close proximity to the KGF-producing cells (data not shown). This expression pattern of KGF and FGFR2 in skin suggests that KGF produced in the dermis acts in a paracrine manner on epidermal keratinocytes.

DISCUSSION

FGFs comprise a family of polypeptide mitogens including aFGF (21), bFGF (22), FGF-3 (Int-2) (23), FGF-4 (Hst) (24, 25), FGF-5 (26), FGF-6 (27), and KGF (28). They are potent mitogens and chemotactic agents *in vitro* for many cells of mesenchymal and epithelial origin and, therefore, have the properties expected of wound cytokines. Furthermore, the

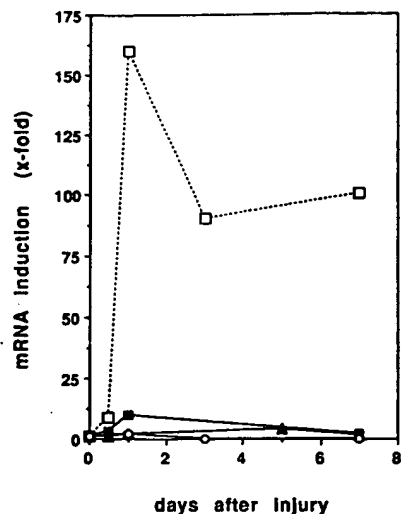


FIG. 3. Induction of aFGF, bFGF, and KGF mRNA expression after injury. The induction of aFGF (■), bFGF (▲), FGF-5 (○), and KGF (□) mRNA expression after injury is shown schematically. The mRNA induction compared with the basal level is shown as a function of time after injury. The increase in FGF mRNA levels was quantified by laser scanning densitometry of the autoradiograms. No expression of FGF-3, FGF-4, and FGF-6 was found in normal skin and within 7 days after injury.

direct application of bFGF to wounds has been shown to have a beneficial effect on accelerating wound healing (2–5).

To investigate the role of endogenous FGFs and FGFRs in wound healing, we have analyzed the mRNA expression of these growth factors in normal skin and during the process of wound healing. Our results show that several members of the FGF family are expressed in normal skin and are induced upon injury. In addition we show that at least three different FGFRs are expressed in normal and wounded skin. These data suggest that multiple FGF-mediated autocrine and paracrine stimulations contribute to the healing of wounds.

Our most striking finding is the extraordinary induction of transcripts encoding KGF within 24 hr after injury. Since KGF had been shown to be a specific and potent growth factor for epithelial cells (28), one might suggest that the induction of its expression during wound healing underlies the migration and proliferation of epithelial cells during this process. This hypothesis is supported by our finding that the induction of KGF expression precedes the onset of epithelial cell proliferation. Furthermore, it persists during the first 7 days after injury, which are characterized by significant migration and proliferation of epithelial cells (29). By *in situ* hybridization we detected expression of KGF mRNA predominantly in some cells below and at the edges of 1-day-old wounds (Fig. 4) and also in later stages of wound healing (days 3–7 after injury; data not shown). These cells most likely represent a distinct population of fibroblasts that might have an important role in wound healing. The detection of KGF transcripts in the dermis of normal and wounded skin is consistent with previous Northern blot results from other investigators who found KGF expression in the dermis but not in the epidermis of normal skin (19). A high-affinity receptor for KGF was recently identified on cultured keratinocytes but not on dermal fibroblasts (20). Therefore, it was suggested that KGF stimulates epithelial cell growth in a paracrine manner.

The receptor for KGF had been shown to be a splice variant of FGFR2, which binds KGF and aFGF with high affinity but bFGF only with low affinity (20, 30). In contrast, other known

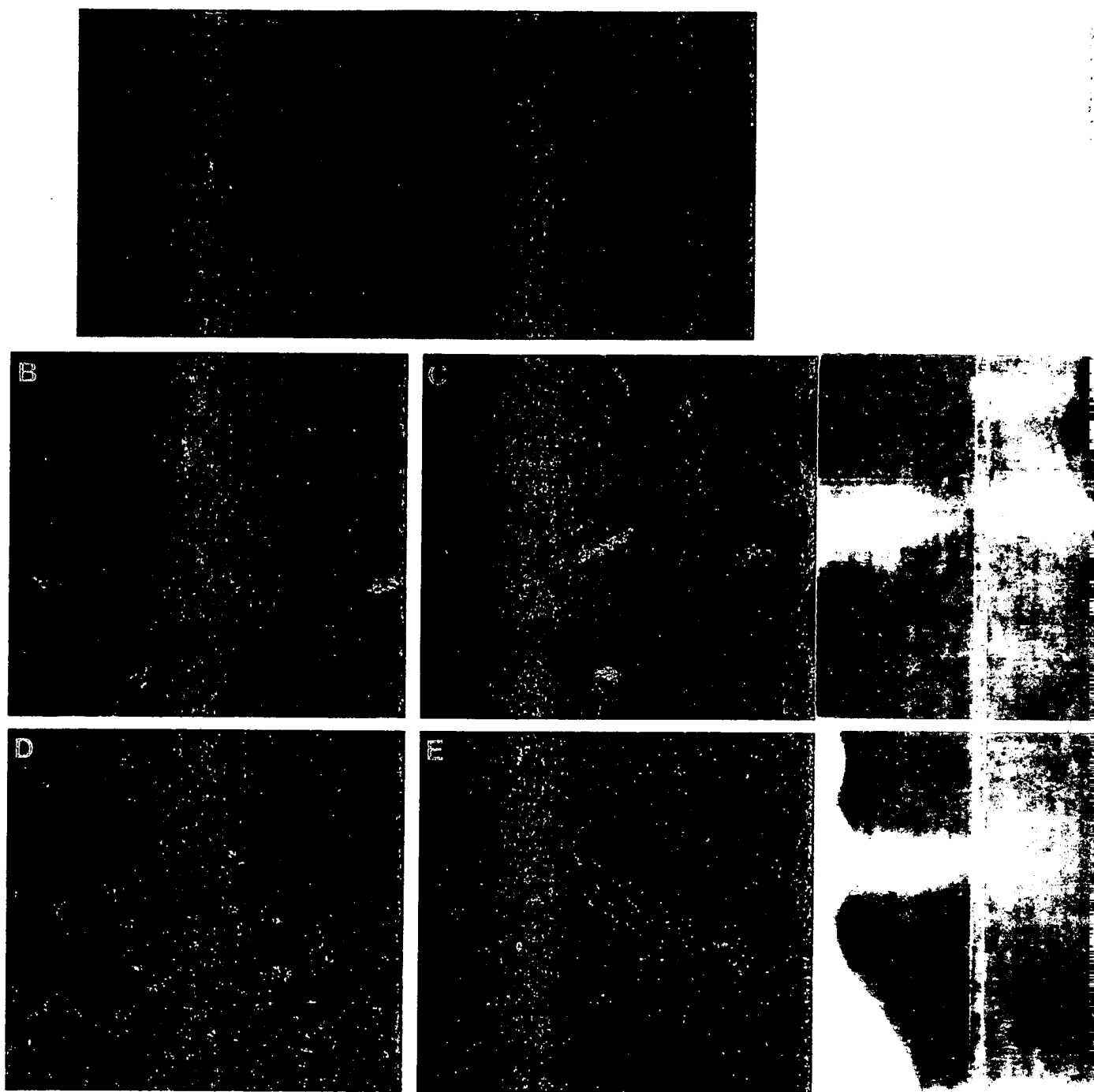


FIG. 4. Localization of FGFR2 and KGF mRNA in normal and wounded skin by *in situ* hybridization. (A) A hematoxylin/eosin stain of half of the wound. The arrowhead on the right indicates the region of normal skin where the photographs in B and C were taken. The arrow in the middle of the wound (left side of A) indicates the area where the photographs in D and E were taken. In A, the letters D, E, Es, and H indicate dermis, epidermis, eschar, and hair follicle, respectively. (B–D) (B and C) Sections from normal mouse back skin. (D and E) Sections from a 1-day-old wound. Sections were hybridized with ^{35}S -labeled riboprobes specific for KGF (B and D) and ^{35}S -labeled riboprobes specific for FGFR2 (C and E). The probes used for hybridization are described in *Materials and Methods*. The silver grains produced by the radioactive probe appear as white dots. They are indicated with arrows in C and D. In B and C the large bright nongranular areas represent artifacts of the autoradiography. Sections were counterstained with hematoxylin/eosin after hybridization. (A, $\times 45$, B–E, $\times 170$.)

FGFR2 splice variants bind aFGF and bFGF with high affinity but do not represent a receptor for KGF (30, 31). Our protection assay data demonstrate that the KGFR form is the

predominant FGFR2 splice variant in skin (data not shown). Therefore, the FGFR2 transcripts which we detected in the epidermis of normal skin and wounds predominantly encode

receptors for KGF which should enable a paracrine stimulation of epidermal fibroblasts by dermally derived KGF.

In contrast to FGFR2, which is found predominantly in the epidermis, FGFR1 mRNA was expressed at highest levels in the dermis of normal and wounded skin (data not shown). A similar pattern of expression of FGFR1 and FGFR2 was recently observed in embryonic skin (32). The differential expression of the two FGFR genes suggests that the encoded proteins mediate different functions. We recently demonstrated that the predominant form of FGFR1 in the skin binds acidic and basic FGF with high affinity but does not represent a receptor for KGF (33). This shows that the major FGFR variants found in the dermis and epidermis have different ligand-binding specificities.

In addition to KGF, we also found induction of aFGF, bFGF, and FGF-5 mRNA expression during wound healing. However, these factors were only expressed at low levels and were induced to a much lesser extent compared with KGF. Among this group of factors expressed at low level, aFGF was induced to the greatest extent. This is of particular interest, since aFGF is the only FGF besides KGF that binds with high affinity to the KGF (30). The induced expression of aFGF might further enhance the proliferation of keratinocytes during wound healing.

Expression of bFGF in skin is consistent with findings of other authors that show the encoded protein in the epidermis (34). In contrast, expression of aFGF and FGF-5 in skin has not been demonstrated so far. The mechanism of action of bFGF and aFGF in normal and wounded skin is presently unclear, since these growth factors lack a signal sequence and, therefore, are not secreted. One might speculate that these mitogens are released by damaged cells upon injury and subsequently act in an autocrine or paracrine manner. In contrast with aFGF and bFGF, the other members of the FGF family, including KGF, are clearly secreted mitogens.

With the exception of bFGF, induction of FGF gene expression seems to be an early event in wound healing, since highest levels of KGF, aFGF, and FGF-5 mRNA were found within 24 hr after injury. Expression of all FGFs subsequently declined, demonstrating the reversibility of the process. Therefore, it seems likely that specific control mechanisms initiate gene expression after injury and subsequently turn off expression after healing. A defect in FGF gene induction might likely be associated with impaired wound healing, whereas a defect in gene suppression could lead to the formation of hypertrophic scars or keloids. In addition, a prolonged induction of FGF gene expression might cause pathologic uncontrolled cell growth which might lead to tumor formation.

In summary, we have provided evidence that FGFs are expressed in normal skin and that their expression is induced during wound healing. The tremendous induction of KGF expression suggests that this mitogen is not only important in the physiological renewal of the epidermis but is of particular importance in the controlled epidermal cell proliferation seen in wound healing.

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